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Stress-Tolerance of *Sinorhizobium* spp. Nodulating Sesbania and Cowpea in Desert Soils

Madhumitha Balasubramani[1], Jyotsna Lakshmi Kumar[1], Nanduri Kameswara Rao[2]*, Neeru Sood[1], Trupti Gokhale[1], Somasundaram Rajeswari[1], Makram Belhaj Fraj[2] and Sanjeet Mishra[3]

[1]Birla Institute of Technology and Science, Pilani – Dubai Campus (BPDC), P.O. Box 345055, Dubai, UAE.
[2]International Center for Biosaline Agriculture (ICBA), P.O. Box 14660, Dubai, UAE.
[3]Advanced Biotechnology Center, P.O. Box 181499, Diera, Dubai, UAE.

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Naturally occurring soil rhizobia were isolated from the root nodules of cowpea and sesbania growing in the desert soils of the United Arab Emirates (UAE). Sequencing of the PCR amplified 16S rDNA identified the rhizobial isolates from cowpea as *Sinorhizobium kostiense* and *S. terangae*, and those from sesbania as *S. meliloti*, *S. arboris* and *Sinorhizobium* sp. nov. *In vitro* growth studies under a range of temperatures, salinities, pH and heavy metals revealed that the UAE strains have greater stress tolerance than the reference strain, *Bradyrhizobium* sp. strain TAL 169. A study of the nodulation ability of the two cowpea strains (*S. kostiense* and *S. terangae*) under saline conditions showed that both the strains were effective in forming root nodules at high salinity (12 dS m^{-1}). To the authors' knowledge, *S. kostiense, S. terangae* and *S. arboris* are reported for the first time from the Arabian Peninsula. Similarly, this study is the first to report symbiotic association of *S. kostiense* and *S. terangae* with cowpea and that of *S. arboris* with sesbania. The tolerance to high levels of abiotic stresses makes these rhizobia highly valuable inoculums to improve the productivity of cowpea and sesbania cultivated in desert areas.

Key words: Cowpea, Heavy metals, pH, Salinity, Sesbania, *Sinorhizobium* spp., Stress tolerance, Temperature.

Rhizobia are free living soil bacteria with the ability to fix atmospheric nitrogen through symbiotic association with leguminous plants. Rhizobia establish themselves in the roots of leguminous plants forming root-nodules, and by fixing atmospheric nitrogen into ammonia, they improve the soil fertility and enhance agricultural productivity. However, a number of factors affect the rhizobium-legume symbiotic relationship. These include the host symbiont compatibility and the physicochemical conditions of the soil, especially temperature extremes, salinity, pH, insufficient or excessive soil moisture, nutrient deficiency, and mineral and heavy metal toxicity[1,2,3].

Salinity is known to decrease plant growth and yield, depending upon the plant species, salinity levels, and ionic composition of the salts. Some legumes (e.g. guar, soybean and cowpea) are known to be more salt tolerant than others (e.g. pea, common bean and lentil). As with the higher plants, increasing salt concentrations can have a detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress. However, rhizobial populations are also known to vary in their tolerance to abiotic stresses[4,5]. While growing stress-tolerant food and forage legumes is recognized as an important adaptation strategy to improve agricultural productivity in marginal environments, identification and inoculation of free living soil rhizobia with genetic potential to tolerate

* To whom all correspondence should be addressed.
E-mail: n.rao@biosaline.org.ae

abiotic stresses is equally important for their effective nodulation and enhanced productivity[6]. The naturally occurring soil rhizobia nodulating the leguminous plants under harsh environments are expected to have higher tolerance to the prevailing abiotic stresses. For instance, local strains of *R. meliloti* isolated from arid soils in Saudi Arabia formed successful symbiosis with *Medicago sativa* under saline conditions[7]. More recently, salt, drought and heavy metals resistance patterns found among the indigenous rhizobial strains were observed to reflect the environmental stresses predominant in their locations, which signifies the importance of using indigenous rhizobial strains for successful and efficient inoculation of the leguminous crops grown under harsh climatic conditions[8,9,10,11].

There are only limited systematic studies to date on the rhizobia associated with the leguminous species in the Arabian Peninsula, characterized by poor and erratic rainfall and high daytime summer temperatures, often exceeding 45°C. Recently, we have isolated naturally occurring soil rhizobia from the root nodules of several leguminous crops irrigated with low-moderately saline water at the experimental station of the International Centre for Biosaline Agriculture (ICBA) in Dubai, UAE. In the present paper, results from a study of the rhizobial isolates for tolerance to abiotic stresses such as high temperatures, salinity, pH and heavy metals are presented.

MATERIALS AND METHODS

The soil at ICBA experimental station (25°13'N and 55°17'E) was fine sand, moderately alkaline (pH 8.22) and strongly calcareous (up to 53% $CaCO_3$) with very low organic matter (<0.5%). The rhizobia used in this study were isolated from the root nodules of cowpea (*Vigna unguiculata* (L.) Walp.) and sesbania (*Sesbania sesban* (L.) Merr.) plants irrigated with low to moderately saline water (2-10 dS m^{-1}). The isolates were purified and characterized based on colony morphology[12]. DNA was extracted using the standard phenol–chloroform method[13] and polymerase chain reaction (PCR) amplification of the 16S rDNA gene was carried out using universal primers (16sF: 5´-AGA GTT TGA TCC TGG CTC AG-3´ and 16sR: 5´- ACG GCT ACC TTG TTA CGACTT-3´)[14]. The PCR products were sequenced using a Big Dye Terminator sequencing kit (Applied Biosystems, USA) as per the manufacturer's protocol and the sequencing data were analyzed using BLAST (Basic Local Alignment Search Tool, maintained by National Centre of Biological Sciences, USA). The result obtained as the maximum identity was taken as the bacterial isolate identification.

Stress tolerance tests

Pure cultures of the rhizobia maintained on Yeast Extract Manitol Agar (YEMA) slants were used for the abiotic stress-tolerance tests. Five isolates, two from cowpea and three from sesbania were tested *in vitro* for their tolerance to elevated temperatures, salinity, pH and heavy metals. Cultures of the five strains (400 µL) were added to 15 ml of Yeast Extract Mannitol Broth (YEMB) contained in test tubes held in a shaking incubator at 150 rpm and growth was recorded every day by measuring the optical density (OD) using a spectrophotometer (Lamda 25, PerkinElmer) at a wavelength of 680 nm over a 5-day period. All tests were performed in triplicate and incubated at 28°C (except for temperature tolerance tests). The relative tolerance of the different strains to any given stress condition was assessed from the growth response, based on the OD measurements on the 5^{th} day of inoculation. *Bradyrhizobium* sp. Strain TAL-169, a slow growing cowpea rhizobium, was used as the reference strain to evaluate the performance of the native strains.

The effect of temperature on growth of the strains was studied by incubating the broth cultures at 25°, 30°, 40° and 50°C. Salinity tolerance was tested by supplementing the culture medium with appropriate amounts of NaCl to obtain electrical conductivities (EC) of 0, 10, 20, 40, 60, 80 and 100 dS m^{-1}. To study the effect of acidity and alkalinity, the pH of the broth prior to inoculation was adjusted to 2, 4, 6, 8, 10 and 12 by adding HCl or NaOH. Five heavy metals (at concentrations indicated in the parenthesis) namely, iron sulphate (100 µg ml^{-1}), zinc sulphate (50 µg ml^{-1}), magnesium sulphate (50 µg ml^{-1}), lead acetate (200 µg ml^{-1}) and copper sulphate (100 µg ml^{-1}) were studied for their effect on growth of the rhizobial strains.

Nodulation study

Cowpea seeds were inoculated with its own rhizobia by soaking for one hour in 100 ml

YEMB cultures adjusted to an OD of 0.5 (4.13×10^5 CFU ml^{-1}). The seeds were dried overnight and sown in 1 lit polyethylene bags containing sterile sand and organic fertilizer in 1:1 ratio. Each strain on inoculated seeds was sown in three sets each of 10 bags (one seed per bag) for establishing the three salinity treatments with EC of 2 (control), 6, and 12 dS m^{-1}. For each level of salinity, an additional set of 10 bags was sown with uninoculated seeds to serve as the negative control. Saline ground water (~20 dS m^{-1}) was blended with fresh water (2 dS m^{-1}) to obtain the higher salinity treatments (6 and 12 dS m^{-1}). The bags were kept on a greenhouse bench and irrigated with fresh water (50 ml per bag) for 17 days, followed by saline water at 6 and 12 dS m^{-1}, depending on the treatment. To ensure adequate levels of infection, 2 weeks old seedlings were re-inoculated with 50 ml of broth cultures of the respective strains. In each treatment, 35 days after sowing, five plants were harvested (which served as replicates) and scored for the number of root nodules and plant growth parameters such as shoot and root lengths, and fresh and dry weights.

The OD data from *in vitro* growth tests, measured on the 5th day of inoculation and the root nodulation data from the greenhouse studies were analysed statistically using GenStat (ver. 7.22DE). A two-way ANOVA was performed to assess the differences in growth of the six rhizobial strains at different temperature, salinity and pH regimes. Principal Components Analysis (PCA) was used to determine the correlations between the rhizobia and the response variables, namely temperature, salinity, pH and heavy metals.

RESULTS AND DISCUSSION

Pure cultures of the rhizobia grown on YEMA medium produced colonies which were white, raised and opaque with mainly smooth (cowpea) or wavy (sesbania) margins. Sequence homologies of 16S rDNA identified the rhizobial isolates from cowpea as *Sinorhizobium kostiense* and *S. terangae.* While two of the three isolates from sesbania were identified as *S. meliloti* and *S. arboris*, the third isolate had a sequence pattern similar to *Sinorhizobium* but different than any of the known species (hence the strain was provisionally named as *Sinorhizobium* sp. nov.). Earlier, *S. arboris, S. terangae* and *S. kostiense* were isolated from root nodules of the leguminous trees, *Acacia senegal* and *Prosopis chilensis* from Senegal, Sudan and Kenya[15,16]. However, this is the first report of their occurrence from the Arabian Peninsula.

Effective symbioses between woody rhizobia from *Acacia* and other trees with crop legumes such as peanut and cowpea was reported earlier[17]. Among the five *Sinorhizobium* strains currently studied, *S. meliloti* and *S. terangae (*ssp. *sesbaniae)* are already known to nodulate sesbania[18, 19]. However, for *S. kostiense* and *S. terangae,* this is the first report of their association with cowpea. Similarly, it is observed for first time that *S. arboris* has the capacity to nodulate sesbania.

In vitro *growth tests*

Analysis of variance revealed significant differences in the growth response of the six strains under the different temperature, salinity and pH regimes. The strains also deferred significantly in their growth response to heavy metals ($P<0.05$) (Figs.1-3).

Temperature tolerance

The effect of temperature on growth of the five *Sinorhizobium* strains together with the reference strain (*Bradyrhizobium* sp. Strain TAL 169) is presented in Fig. 1A. All the strains, including the reference strain, exhibited good growth at 30°C and an increase in temperature led to a sharp decline in growth, except in *S. kostiense* which showed the maximum growth at 40°C. All the strains had marginal growth at 50°C, with the OD ranging from 0.005 (*S. meliloti*) to 0.046 (*S. kostiense*). As can be seen from Fig. 1A, *S. kostiense* also grew well at 20°C, indicating its tolerance to a wider range of temperatures compared to other strains. In general, *S. terangae* exhibited low-growth at all temperatures, the mean maximum OD being 1.2 at 30°C, whereas the maximum OD values for the other strains were in the range of 2.0-2.5 (Fig. 1A).

It is well established that growth and survival of rhizobia in soils are adversely affected by high soil temperatures. For most rhizobia, while the optimum temperature range for growth and nodulation is reported to be 25-30°C[6,20,21], strains of cowpea rhizobium from the hot and dry environment of the Sahel Savannah were reported to grow well at 40°C[22]. In the present study, *S. kostiense* isolated from cowpea showed similar tolerance to temperature. Also, in case of sesbania, strains tolerating up to 44°C temperatures were identified[4,23]. However, all the three sesbania strains reported here were found to be sensitive to high temperature stress.

Salinity tolerance

The growth response of the six rhizobial strains to different NaCl concentrations is presented in Fig.1B. Overall, all the strains showed good growth between 5 and 20 dS m^{-1}, except the reference strain (TAL 169). In term of salt-tolerance, *S. meliloti* and *S. arboris* grew well even at 40 dS m^{-1}, compared to others strains and increase in salinity beyond 40 dS m^{-1} led to a drastic reduction in growth of all strains, including *S. meliloti* and *S. arboris*. At 60 and 80 dS m^{-1} salinity, *S. terangae* and *S. kostiense* exhibited low growth (OD between 0.2 and 0.5), in contrast to the minimal growth observed in other strains. Interestingly, all the strains except TAL 169 showed better growth at 5 dS m^{-1} salt concentrations than at 0 dS m^{-1} (0.002 dS m^{-1} in effect, since YEMB contains 0.1 g l^{-1} of NaCl), which shows that some amount of salt promotes growth of the native rhizobia.

Rhizobial cultures are known to show marked variation in salt tolerance. In general, growth of a number of rhizobia was inhibited by low salt concentrations, although some rhizobia including *S. meliloti*, were found to be tolerant to high salinity[6,7,24]. Among the cowpea strains, growth under *in vitro* conditions was observed to be heavy at 0-0.005 M NaCl concentrations (ca. 0-0.4 dS m^{-1}), but lighter at 0.2 M (ca. 16.8 dS m^{-1})[25]. However, some strains were reported to tolerate up to 5.5% NaCl (ca.78.5 dS m^{-1})[26]. More recently, *Rhizobium* sp. associated with *Vigna marina*, a wild legume growing in sandy seashores was also found to have tolerance up to 1000 mM of NaCl (80.6 dS m^{-1})[27]. In the present study, among the two cowpea strains, *S. kostiense* and *S. terangae* exhibited good growth up to a salinity of 20 dS m^{-1} and further increase in salinity resulted in sharp reduction in growth. Nonetheless, unlike other strains, both these strains survived at higher salinities (60 and 80 dS m^{-1}) as evident from the OD measurements.

In case of the rhizobial strains associated with sesbania, tolerance of up to 600 mM (ca 50.4 dS m^{-1}) was reported under *in vitro* conditions[23]. In our study, *S. meliloti* and *S. arboris* from sesbania tolerated up to 40 dS m^{-1} salinity, but further increase in salinity resulted in severe reduction in growth (Fig.1B).

pH tolerance

All the native strains showed more or less a similar trend in growing better under alkaline rather than acidic conditions (Fig. 1C). Thus, the optimal pH for growth varied between 8 and 10. The reference strain (TAL 169) however showed the maximum growth at 6 pH. Averaged over strains, growth was maximum at a pH of 8 and no growth was observed at pH 2, 4. In contrast to other strains, *S. terangae* and *S. kestiense* exhibited low growth at 12 pH, with OD values of 0.72 and 0.36, respectively. Overall, *S. terangae* had a low growth rate, the maximum OD being about 1.5 (at pH 8), compared to the values of 3-3.5 for other strains.

In general, the optimum pH for rhizobial growth was reported to be between six and seven[6,25]. However, Rhizobia with a higher tolerance to acidity and alkalinity have been identified[28,29,30,31]. In sesbania, strains of *Rhizobium* sp. That survived at pH 11 and 12 and in cowpea strains which are tolerant to pH values as low as 4.0 have been isolated[4,26]. It is also known that strains of some species vary widely in their pH tolerance. Thus, while most strains of *R. meliloti* were sensitive to acid stress[1,32], some strains were found to perform well at pH 5.0 to 5.5[24, 33].

Heavy metals tolerance

The effect of heavy metals on growth of the rhizobia is presented in Fig. 2. In general, all the strains studied were found to be sensitive to Cu and Zn, though TAL 169, *S. terangae* and *S. kostiense* were the most affected among others. Fe, Mg and Pb had variable effects on the growth of various strains. Thus, at the same concentration of heavy metal and in comparison with other strains, the growth of Tal 169, *S. terangae* and *S. kostiense* was relatively less indicating their sensitivity. Inherent differences in tolerance of *S. meliloti* strains to heavy metal concentrations were also reported[8,34]. It should be noted that soil pH besides directly affecting the survival of the rhizobia, also influences the heavy metal concentrations and thereby affects the growth and nodulation ability of the rhizobia. For instance, low soil pH can result in increased solubility of certain metal ions (e.g. Al, Cu and Zn) to the point where toxic levels are reached. In contrast, alkaline soil condition can lead to deficiencies as the metals become increasingly unavailable[35].

S. arboris strains from Sudan had a maximum growth temperature of 41-43°C, and showed tolerance to 3% (w/v) NaCl (ca. 42.8 dS m^{-1}) and heavy metals, except Al[15]. With *S. kostiense*, the maximum growth temperature was found to be 38-40°C, the species showed tolerance to only 1% (w/v) NaCl (ca. 14.3 dS m^{-1}) and sensitivity to heavy metals except Cu and Pb[15]. Thus, in terms of tolerance to temperature and salinity stresses, the UAE strains *S. kostiense* and *S. arboris* were similar to the Sudanese strains. However, while *S. arboris*

1(A)
1(B)
1(C)

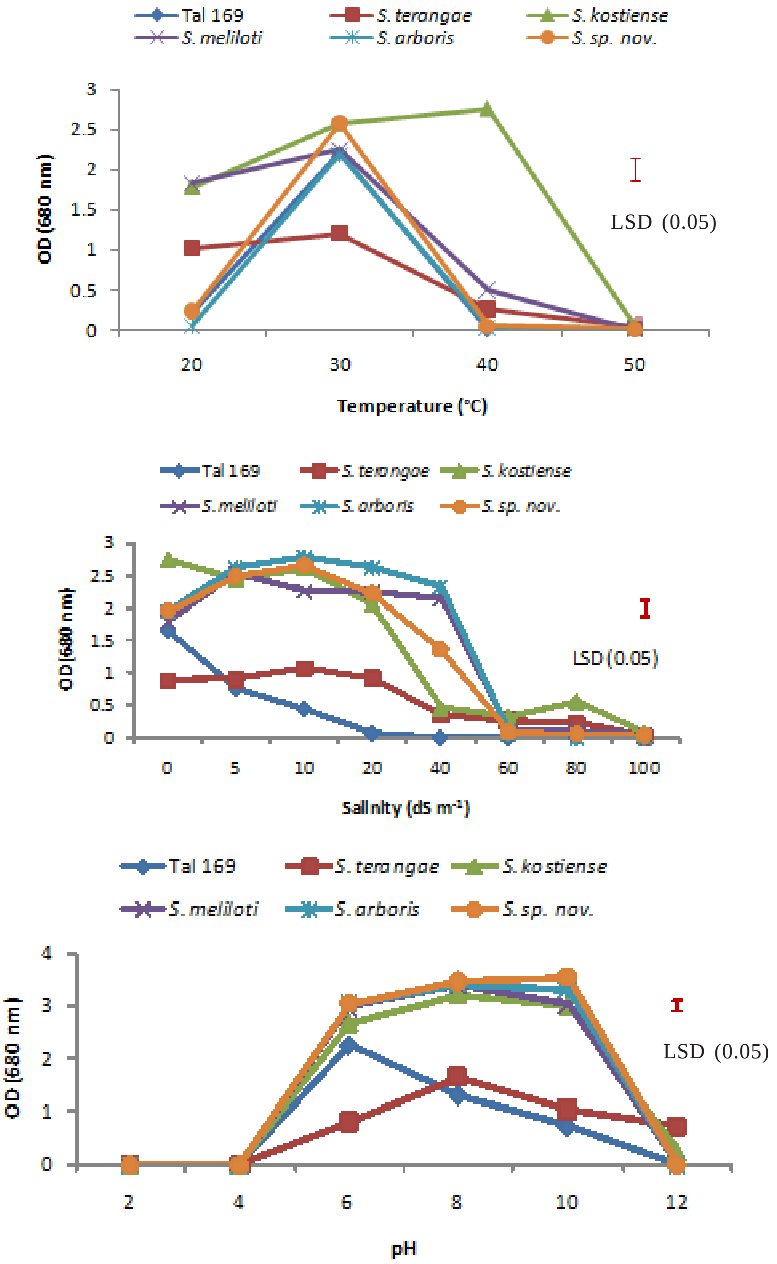

Fig. 1. Effect of temperature (A), salinity (B) and pH (C) on growth of five *Sinorhizobium* spp. and the reference strain *Bradyrhizobium* sp. Strain TAL 169

from Sudan was more tolerant of the heat stress, *S. kostiense* from the UAE was found to have considerably higher tolerance to salinity than the Sudanese strain.

Nodulation

The results of seed inoculation are presented in Table 1. While the uninoculated plants remained free of nodules, salinity had variable effect on nodulating ability of the two cowpea stains. While the reference strain TAL-169 failed to form any root nodules when water salinity was above 6 dS m^{-1}, *S. kostiense* and *S. terangae* were effective in nodulating even at 12 dS m^{-1} salinity. Between the two strains, *S. kostiense* was more efficient as the 5-week old seedlings had an average of 26.2 nodules per plant compared to 2.0 per plant with *S. terangae*. Interestingly, neither the salinity treatment nor the nodulation did show any effect on plant growth. Thus, no significant differences were found in shoot and root length between treatments. The differences between treatments for fresh and dry weight were also insignificant (data not presented).

Table 1. Nodulation in cowpea inoculated with different strains of rhizobia and irrigated with saline water

Salinity	Strain				
(dS m^{-1})	Uninoculated	TAL 169	*S. terangae*	*S. kostiense*	Mean
	Nodules/plant				
2	0.0	1.0	6.4	55.2	15.7
6	0.0	1.2	9.0	41.2	12.9
12	0.0	0.0	2.0	26.2	7.1
Mean	0.0	0.7	5.8	40.9	11.9
LSD (5%)			13.7		

The depressive effect of NaCl on nodulation was also observed earlier in several crops, including cowpea and sesbania. In cowpea, while the number and size of nodules remained more or less the same between 0.005-0.050 M of NaCl (ca. 0.4-4.2 dS m^{-1}) there was simultaneous reduction of both at higher concentrations (0.100-0.200 M, i.e. 8.3-16.7 dS m^{-1})[25]. In sesbania, salinity decreased the number of nodules per plant but increased the size of nodules[36]. On the other hand, both the number and size of the nodules per plant and their fresh and dry weights decreased with an increase in salinity[37]. In the present study, nodulation was found to be more sensitive to salinity than plant growth, as also observed in soybean[38]. In *Leucaena leucocephala*, NaCl

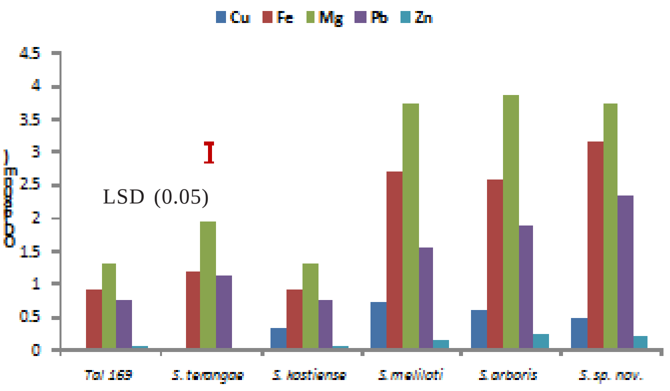

Fig. 2 . Effect of heavy metals on growth of five *Sinorhizobium* spp. and the reference strain *Bradyrhizobium* sp. Strain TAL 169

treatment did not show any adverse effects on plant growth during the first seven weeks of treatment, but distinct differences began to occur after 14 weeks of treatment[39]. It is also possible that the organic fertilizer used in the planting medium could have masked beneficial effects of the rihizobia on plant growth in the present study.

Principal components analysis

The results of the principal components analysis (PCA) are shown in Fig. 3. It can be seen that PC_1 and PC_2, together contributed to 98% of the total variation present among the strains taking into account all four stresses simultaneously. PC_1 accounted for 75% of the variability and was most influenced by salinity, pH and heavy metals, while PC_2 which accounted for 23% of the variability was most influenced by temperature. *S. kostiense* displayed high positive coefficients of both components, in contrast total 169 and *S. terangae*, which have negative coefficients. The analysis showed that *S. kostiense* was most adapted to temperature and *S. meliloti* to salinity, while *S. arboris* and *Sinorhizobium* sp. nov. were most adapted to pH and heavy metals.

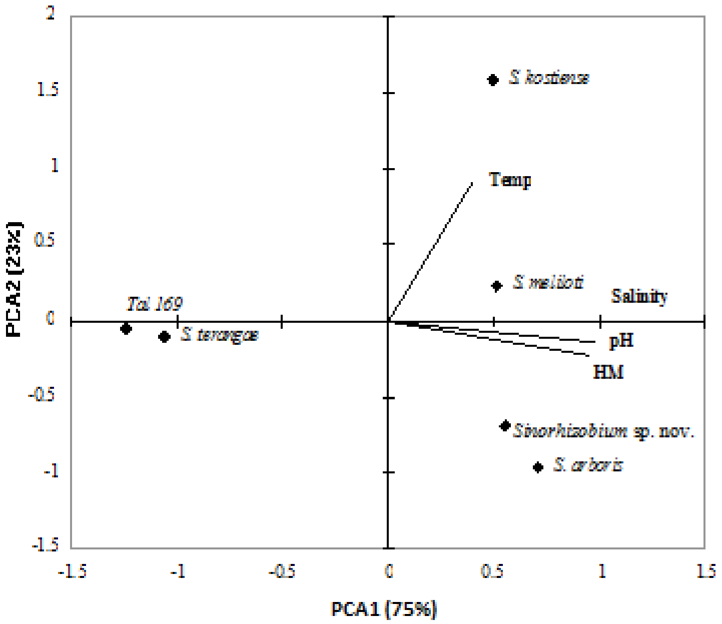

Fig. 3. BiPlot of principal component analysis of the variates, temperature (Temp), salinity, pH and heavy metals (HM) and the six rhizobial strains shown

CONCLUSIONS

The results from this study show that the rhizobia isolated from the sandy soils at ICBA experimental station have developed tolerance to abiotic stresses such as high temperatures, salinity and alkalinity, which enable them to survive under the extreme desert conditions. Among the five native strains studied, while the optimum temperature for growth was found to be 30°C, *S. kostiense* depicted good growth at 40°C. *S. meliloti* and *S. arboris* were the most halotolerant, exhibiting good growth even at salinities as high as 40 dS m^{-1}. It is significant to note that the native rhizobial strains were also tolerant to highly alkaline conditions as evident from their survival at a pH of 10.

Since cowpea and sesbania seeds used for planting were not inoculated with rhizobia and there is no previous history of cultivation of any leguminous crops in the same fields, it is obvious that the rhizobia are naturally occurring native strains surviving and persisting in harsh desert environments. The possession of high levels of stress tolerance might be an evolutionary significance for the survival of the rhizobia under adverse conditions. Alkaline soil, high temperature and high salinity are major constraints for plant growth and productivity in arid and hyper-arid areas. The tolerance to environmental stresses, especially high salinity and temperature, make these rhizobia highly valuable inoculums to improve the productivity of compatible leguminous crops cultivated in marginal and salt-affected soils.

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REFERENCES

1. Brockwell, J., Pilka, A., Holliday, R.A. Soil pH is a major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non-cultivated soils of New South Wales. *Aust. J. Exp. Agric.*, 1991; **31**: 211-219.
2. Thies, J.E., Woomer, P.L., Singleton, P.W. Enrichment of *Bradyrhizobium* spp. populations in soil due to cropping of the homologous host legume. *Soil Biol. Biochem.*, 1995; **27**: 633-636.
3. Wolf, J.J., Rohrs, J. The influence of physical soil conditions on the formation of root nodules of *Melilotus officinalis* in the montane zone of Rocky Mountain National Park. *Eur. J. Soil Biol.*, 2001; **37**: 51-57.
4. Kulkarni, S., Surange, S., Nautiyal, C.S. Crossing limits of Rhizobium existence in extreme conditions. *Curr. Microbiol.*, 2000; **41**: 402-409.
5. Wei, G.H., Yang X.Y., Zhang, Z.X., Yang, Y.Z., Lindstrom,K. Strain Mesorhizobium sp. CCNWGX035; A stress tolerant isolate from Glycyrriza glabra displaying a wide host range of nodulation. *Pedosphere*, 2008; **18**: 102-112.
6. Zahran, H.H. Rhizobium-legume sysmbiosis and nitrogen fixation under severe conditions and in arid climates. *Microbiol. Mol. Biol. Rev*., 1999; **63**: 968-989.
7. Mashhady, A.S., Salem, S.H., Barakh, F.N., Heggo, A.M. Effect of salinity on survival and symbiotic performance between *Rhizobium meliloti* and *Medicago sativa* in Saudi Arabian soils. *Arid Soil Res. Rehab*., 1998; **12**: 3-14.
8. Abdel-Salam, M.S., Ibrahim, S.A., Abd-El-Halim, M.M., Badawy, F.M., Abo-Aba, S.E.M. Phenotypic characterization of indigenous Egyptian Rhizobial strains for abiotic stresses performance. *J. Am. Sci.*, 2010; **6**: 498-503.
9. Elboutahiri, N., Thami-Alami, I., Udupa, S.M. Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco. *BMC Microbiol*., 2010; **10**: 10-15.
10. Essendoubi, M., Brhada, F., Eljamali, J.E., Filali-Maltouf, A., Bonnassie, S., Georgeault, S., Blanco, C., Jebbar, M. Osmoadaptative responses in the rhizobia nodulating *Acacia* isolated from south-eastern Moroccan Sahara. *Environ. Microbiol.*, 2007; **9**: 603-11.
11. Diouf, D., Samba-Mbaye, R., Lesueur, D., Ba, A.T., Dreyfus B., de Lajudie P., Neyra M. Genetic diversity of *Acacia seyal* Del. rhizobial populations indigenous to Senegalese soils in relation to salinity and pH of the sampling sites. *Microb. Ecol*., 2007; **54**: 553-566.
12. Sharma, S.R., Rao, N.K.,Trupti, S.G., Ismail, S. Isolation and characterization of salt-tolerant rhizobia native to the desert soils of United Arab Emirates. *Emir. J. Food Agric.*, 2013; **25**:102-108.
13. Davis, G.L., Dibner, D.M., Battey, F.J. Preparation of DNA from Eukaryotic cells: General method. In: *Basic Methods in Molecular Biology* (Davis LG, Leonard G, eds). Elsevier Science Publishing, New York, 1989; pp 44 46.
14. Bricker, B.J., Ewalt, D.R., MacMillan, A.P., Foster, G., Brew, S. Molecular Characterization of *Brucella* strains isolated from marine mammals. *J. Clin. Microbiol*., 2000; **38**: 1258-1262.
15. Nick, G., de Lajudie, P., Eardly, B.D., Suomalainen, S., Paulin, L., Zhang, X., Gillis, M., Lindström, K. *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. *Int. J. Sys. Bacteriol*., 1999; **4**: 1359-1368.
16. de Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Ney, M., Collins, M.D., Dreyfus, B., Kersters, K., Gillis, M. Polyphasic Taxonomy of Rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int. J. Sys. Bacteriol*., 1994; **44**: 715-733.

17. Wange, S.S. Response of groundnut (*Arachis hypogaea* L.) to inoculation with *Rhizobium* strains isolated from wild arboreal legumes. *J. Appl. Microbiol. Biotech*., 1989; **5**: 135-141.
18. Sharma, R.S., Mohmmed, A., Mishrab, V, Babu C.R. Diversity in a promiscuous group of rhizobia from three Sesbania spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. *Res. Microbiol*., 2005: **156**: 57-67.
19. Loret, G., Méar, N., Lorquin, J., Dreyfus, B., de Lajudie, P., Rosenberg, C., Boivin, C. Nod factor thin-layer chromatography profiling as a tool to characterize symbiotic specificity of rhizobial strains: Application to *Sinorhizobium saheli*, *S. teranga*, and *Rhizobium* sp. strains isolated from *Acacia* and *Sesbania*. *Mol. Plant Microbe Interact*. 1996; **9**: 736-747.
20. Graham, P.H. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Can. J. Microbiol*., 1992; **38**: 475-484.
21. Ali, S.F., Rawat, L.S., Meghvansi, M.K., Mahna, S.K. Selection of stress-tolerant rhizobial isolates of wild legumes growing in dry regions of Rajasthan, India. *ARPN J. Agric. Biol. Sci*., 2009; **4**: 13-18.
22. Eaglesham, A.R.J., Ayanaba, A.: Tropical stress ecology of rhizobia, root nodulation and legume nitrogen fixation. In: *Selected Topics in Biological Nitrogen Fixation* (Subba Rao NS, ed) Oxford/ IBH Publishing, 1984. pp 1-35.
23. Gopalkrishnan, S., Girish, A.G. Physiological and biochemical characteristics of fast growing rhizobia of *sesbania bispinosa* in the *in vitro* condition. *Legume Res*., 1999; **22**: 227-232.
24. Yan, A.M., Wang, E.T., Kan, F.L., Tan, Z.Y., Sui, X.H., Reinhold-Hurek, B.,Chen, W.X. *Sinorhizobium meliloti* associated with *Medicago sativa* and *Melilotus* spp. in arid saline soils in Xinjiang, China. *Int. J. Sys. Evol. Microbiol*., 2000; **5**: 1887-1891.
25. Mensah, J.K., Esumeh, F., Iyamu, M., Omoifo, C. Effects of different salt concentrations and pH on growth of *Rhizobium* sp. and a cowpea-*Rhizobium* association. *American-Eurasian J. Agric. Environ. Sci*., 2006; **1**: 198-202.
26. Mpepereki, S., Makonese, F., Wollum, A.G. Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* 1997; **22**: 275-292.
27. Elanchezhian, R., Rajalakshmi, S., Jayakumar, V. Salt tolerance of rhizobium species associated with *Vigna marina*. *Indian J. Agric. Sci*., 2009; **79**: 980-985.
28. Graham, P.H., Draeger, K., Ferrey, M.L., Conroy, M.J., Hammer, B.E., Martinez, E., Naarons, S.R., Quinto, C. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR 1899. *Can. J. Microbiol*., 1994; **40**: 198-207.
29. Appunu, C., Reddy, L.M.L., Reddy, C.V.C.M., Sen, D., Dhar, B. Symbiotic diversity among acid-tolerant bradyrhizobial isolates with cowpea. *J. Agric. Sci*., 2009; **4**: 126-131.
30. Arun, A.B., Sridhar, K.R. Growth tolerance of Rhizobia isolated from sand dune legumes of the southwest coast of India. *Eng. Life Sci*. 2005; **5**: 134-138.
31. Pongsilp, N., Nuntagij, A. Selection and characterization of mungbean root nodule bacteria based on their growth and symbiotic ability in alkaline conditions. *Suranaree J. Sci. Technol*., 2007; **14**: 277-286.
32. Tiwari, R.P., Reeve, W.G., Glenn, A.R. Mutation conferring acid sensitivity in the acid tolerant strains *Rhizobium meliloti* WSM419 and *Rhizobium leguminosarum* biovar. *viciae* WSM 710. *FEMS Microbiol. Lett*., 1992; **100**: 107–112.
33. Clarke, L.M., Dilworth, M.J., Glenn, A.R. Survival of *Rhizobium meliloti* WSM 419 in laboratory culture: effect of combined pH shock and carbon. *Soil Biol. Biochem.*, 1993; **25**: 1289-1291.
34. Milièiæ, B., Deliæ, D., Stajkoviæ, O., Rasuliæ, N., Kuzmanoviæ, D., Jošiæ, D. Effects of heavy metals on rhizobial growth. *Roum. Biotechnol. Lett.*, 2006; **11**: 2995-3003.
35. Hirsch, A.M. How *Rhizobia* survive in the absence of a legume host, a stressful world indeed. In: *Symbioses and Stress: Joint Ventures in Biology* (Seckbach J, Grube M, eds) Springer Science+Business Media, 2010; pp 375–391.
36. Mirza, J.I., Tariq, R. Effect of sodium chloride on growth and nodulation of *Sesbania seban*. *Pak. J. Bot.*, 1992; **24**: 209-212.
37. Mahmood, A., Athar. M., Qadri. R., Mahmood, N. Effect of NaCl salinity on growth, nodulation and total nitrogen content in *Sesbania sesban*. *Agric Conspec. Sci.*, 2008; **73**: 137-141.
38. Elsheikh, E.A.E., Wood, M. Effect of salinity on growth, nodulation and nitrogen yield of chickpea. *J. Exp. Bot.*, 1990; **41**: 1263-1269.
39. Anthraper, A., Du Bios, J.D. The effect of NaCl on growth, N_2 fixation (acetylene reduction) and percentage total nitrogen in *Leucaena leucocephala* (Leguminosae) var. K-8-1. *Am. J. Bot*., 2003; **90**: 683-692.